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Promising Immunomodulatory Effects of Selected Strains of Dairy Propionibacteria as Evidenced *In Vitro* and *In Vivo*[▽][†]

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Immunomodulatory properties of 10 dairy propionibacteria, analyzed on human peripheral blood mononuclear cells (PBMCs), revealed a highly strain-dependent induction of anti-inflammatory cytokine interleukin 10 (IL-10). Two selected strains of *Propionibacterium freudenreichii* showed a protective effect against two models of colitis in mice, suggesting a probiotic potential predicted by immune-based selection criteria for these cheese starter bacteria.

Selected immunomodulatory probiotic bacteria can counteract inflammation of the intestine through multiple regulatory activities and may be either complementary or an alternative to conventional treatments toward inflammatory bowel disease (IBD), a growing health concern in developed countries. Probiotic strains such as lactobacilli and bifidobacteria are able to induce anti-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs) *in vitro* and were shown to exert efficient anti-inflammatory effects on colitis *in vivo* (9, 26). However, little is known about the immunomodulatory potential of highly consumed starter bacteria such as dairy propionibacteria. Consumption of fermented products has an impact on immune system function (25), and the bacterial content of these products is responsible for immunomodulation (6, 10). Dairy propionibacteria display various probiotic properties either similar to or distinct from those reported for probiotic bifidobacteria and lactic acid bacteria (3). Although an anti-inflammatory potential of a few dairy propionibacterium strains was occasionally suggested *in vitro* (15) or in animals (22, 24, 31), no reliable observation was established in terms of strain variability and of specific mechanisms involved. In addition, supplementation with dairy propionibacteria in human randomized, placebo-controlled, double-blind trials has mainly concerned mixtures comprising probiotic bacteria assigned to genera other than *Propionibacterium* but rarely with propionibacteria alone (16). Thus, because of synergistic effects, it is not possible to attribute observed health benefits to a specific bacterium *per se* within the mixtures.

While some criteria such as adhesion and related immunomodulation through epithelial cells may affect further performance *in vivo*, depending on the expected probiotic effect (13, 21, 27), each probiotic strain is preferably characterized for its immune activity on immunocompetent cells (2, 19, 21, 33) or in cell coculture models before being proposed for clinical application (23). The results obtained may help to evaluate the specific potential for the strain(s) to induce immune responses of the Th1 or the anti-inflammatory type. Here we questioned whether this *in vitro* approach is applicable to propionibacteria and whether results match with *in vivo* pathological animal models which mimic human gastrointestinal diseases and immune disorders.

***In vitro* immunomodulatory-based screening.** By using a previously described *in vitro* assay of cytokine release by human PBMCs (8, 9), we evaluated the cytokine induction pattern for a set of 10 dairy propionibacteria. This included *P. freudenreichii* CIRM-BIA1, CIRM-BIA118, CIRM-BIA456, ITGP18, ITGP20, SI48, SI41, LSIP11, and LSIP23 and *P. jensenii* CIRM-BIA455. These strains were provided by the culture collections CIRM-BIA (INRA STLO), ITG (Actilait, Rennes, France), or Laboratoires STANDA (Caen, France) and grown at 30°C in YEL medium until early stationary phase in microaerophilic conditions (18). A first screening was performed on these 10 strains, using PBMCs from four distinct donors. Tumor necrosis factor alpha (TNF-α), interleukin (IL)-10, gamma interferon (IFN-γ), and IL-12p70 cytokines were measured by enzyme-linked immunosorbent assay (ELISA) (9). It revealed a highly strain-dependent induction of IL-10, covering the range (150 to 2,500 pg/ml) generally obtained with reference strains of lactobacilli (*Lactobacillus acidophilus* NCFM and *Lactobacillus salivarius* Ls33, kindly provided by Danisco), lactococci (*Lactococcus lactis* MG1363, Institute of Food Research, Norwich, United Kingdom), and bifidobacteria (*Bifidobacterium longum* 5336, from Morinaga Milk Industry Ltd.)
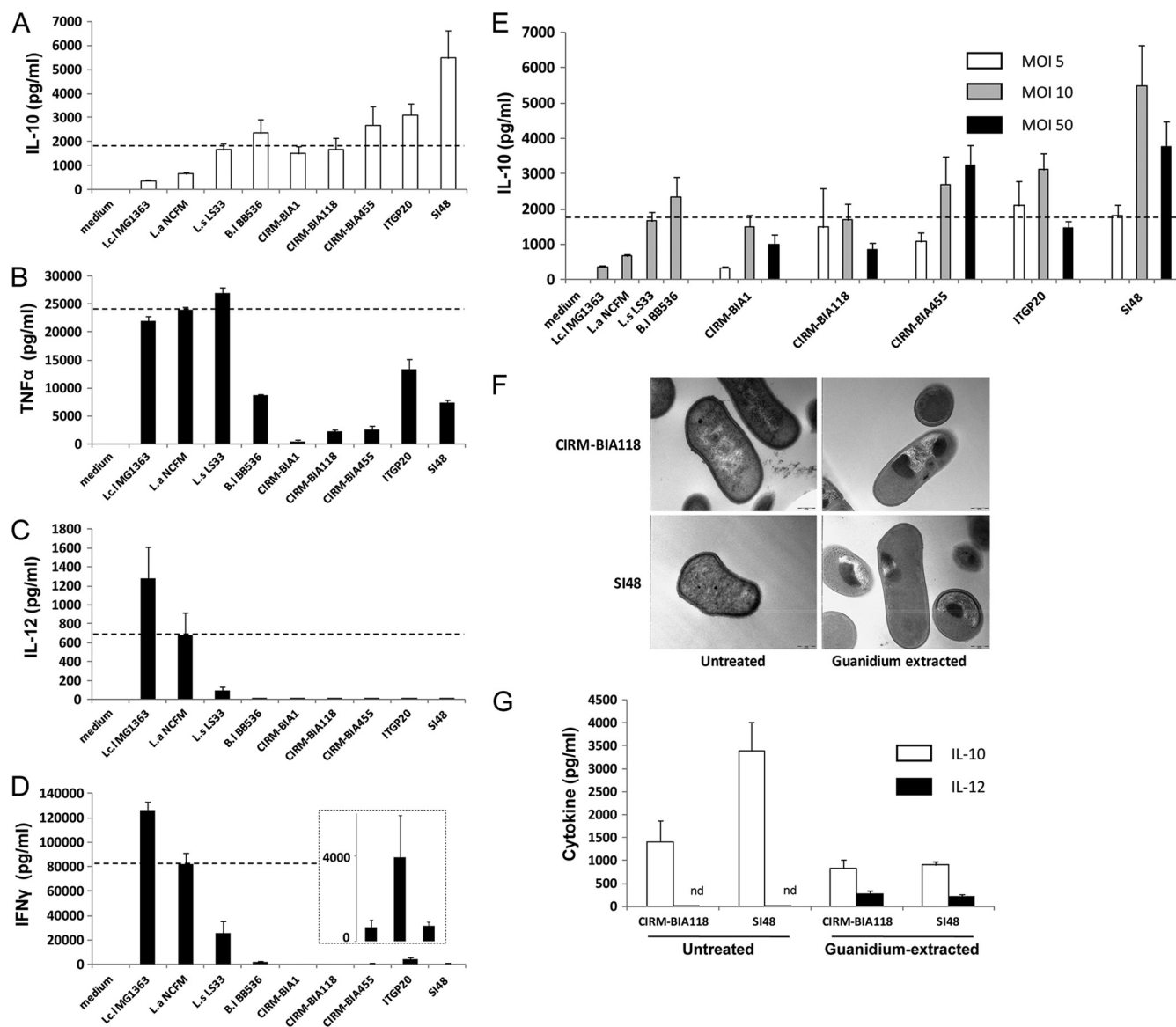
FIG. 1. *In vitro* immunomodulatory-based screening. Comparative anti-inflammatory IL-10 (A) and proinflammatory TNF-α (B), IL-12 (C), and IFN-γ (D) cytokine responses of human PBMCs for four reference and five propionibacterium strains at a bacterial density corresponding to a multiplicity of infection (MOI) of 10. (E) IL-10 response at distinct dosing (MOI of 5, 10, and 50). (F) Anti-inflammatory role of surface layer protein(s). Transmission electron microscopy appearance of untreated (left panel) and guanidium chloride-treated *P. freudenreichii* BIA118 and SI48, showing removal of specific surface layer. (G) IL-10 and IL-12 response of *P. freudenreichii* BIA118 and SI48 and the corresponding extracted bacteria (MOI of 10 for all strains). Immunocompetent cells were stimulated by bacteria for 24 h and collected supernatants analyzed by ELISA. Data are expressed in pg/ml as means and standard deviations of results for four distinct healthy blood donors (the dashed lines indicate the cutoff *in vitro* threshold of the corresponding strain previously shown to be protective *in vivo* [9]; dashed window for panel D corresponds to a ×25 magnification).

cultivated and analyzed as recently described (8). Briefly, we observed strong, moderate, and weak inducers of IL-10 for propionibacteria at a bacterium/host cell ratio of 10:1 (multiplicity of infection [MOI] of 10; data not shown). We then selected five strains to verify the dose dependency of the IL-10 induction (MOI of 5, 10, and 50), confirming that the MOI-of-10 dose was the most appropriate for screening purposes (Fig. 1A and E). *P. freudenreichii* ITGP20 and SI48 were found to be the most anti-inflammatory strains, while *P. jensenii* BIA455 and *P. freudenreichii* BIA118 were less anti-inflamma-

tory, although still more than the *P. freudenreichii* BIA1 type strain (Fig. 1A). Interestingly, for all the *P. freudenreichii* strains tested, release of proinflammatory mediators was very low: weak for TNF-α (Fig. 1B) and almost undetectable for IL-12 and IFN-γ (Fig. 1C and D). The latter cytokine levels were even well below the levels described for strains that offer little or no protection in *in vivo* inflammation models (9) (see cutoff levels in Fig. 1B to D), while IL-10 levels were clearly at or above the cutoff level that generally allows protection in a trinitrobenzene sulfonic acid (TNBS)-induced colitis model (9)
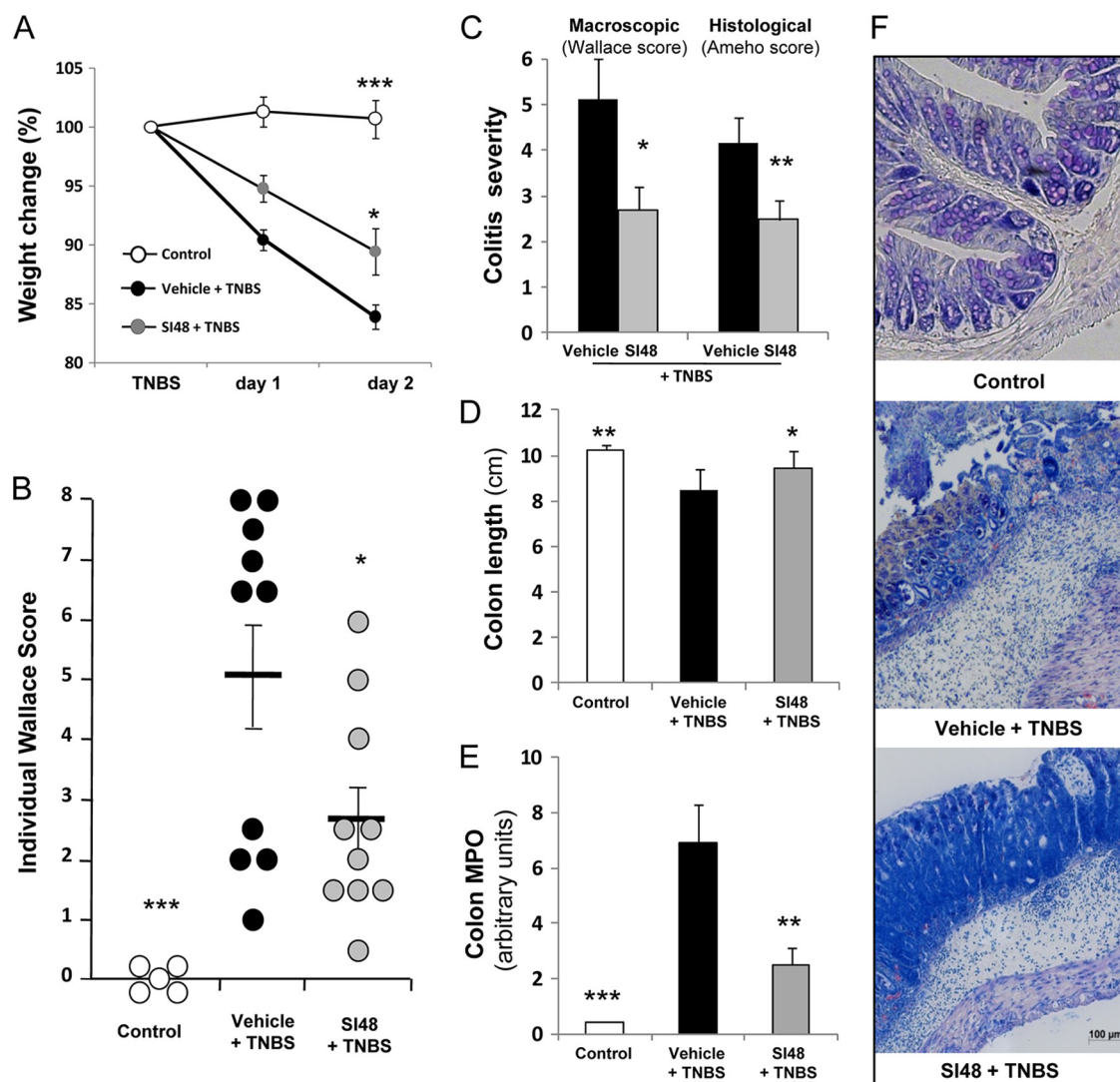
FIG. 2. Protective effect of 5 days of oral treatment with *P. freudenreichii* SI48 (5×10^8 CFU per day) on TNBS colitis. (A) 2-day body weight loss (percentage of initial weight) for healthy control mice (empty circles), vehicle-TNBS-treated animals (black circles), and SI48-fed mice (gray circles). (B) Individual macroscopic disease scores (Wallace score). (C) Macroscopic and histological damage scores (Ameho score). (D) Changes in colon length (cm). (E) Colonic MPO activities. Results for panels B to E were all recorded 48 h after induction of colitis. Results are expressed as means ± standard errors of the mean ($n = 10$ per group); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus vehicle by Mann-Whitney U test or the Student t test where appropriate. (F) Representative histological sections from an untreated mouse (upper panel) and from mice after induction of TNBS colitis following treatment either with vehicle (middle panel) or with *P. freudenreichii* SI48 (lower panel); May-Grünwald- and Giemsa-stained 5-µm paraffin sections, ×40.

(Fig. 1A). This interesting absence of proinflammatory cytokines was confirmed at the transcriptional level for high, intermediary, and lower anti-inflammatory strains. Quantitative reverse transcriptase PCR (RT-PCR) was performed on RNA from PBMCs isolated 4.5 h after induction (see Fig. S1A, S1B, and S1C in the supplemental material) and matched with ELISA quantifications of proteins of the corresponding donors in 24-h supernatants (see Fig. S1D, S1E, and S1F in the supplemental material). This investigation confirmed the large diversity in the immunomodulatory profiles among propionibacteria, quantified directly by the IL-10 cytokine, rather than the traditional IL-10/IL-12 ratio, which, obviously, is inappropriate here.

Efficacy of selected candidates in animal models. The strain *P. freudenreichii* SI48, showing a particularly high anti-inflammatory profile, was further tested in a 5-day prophylactic oral treatment (5×10^8 CFU per mouse and per day or the corresponding vehicle) in the TNBS-induced acute colitis model in mice (8). This "gold standard" model was used as described previously (female BALB/c mice, from Charles River, France, aged 7 weeks, $n = 10$ per group). *P. freudenreichii* SI48 significantly lowered the colitis-associated weight loss at day 2 (10.5% versus 16%; $P < 0.05$; Fig. 2A), confirmed by significant changes of the macroscopic (Fig. 2B and C, corresponding to 45% of protection; $P < 0.05$) and histological scores (Fig. 2C and F). Consistently, inflammatory markers such as colon
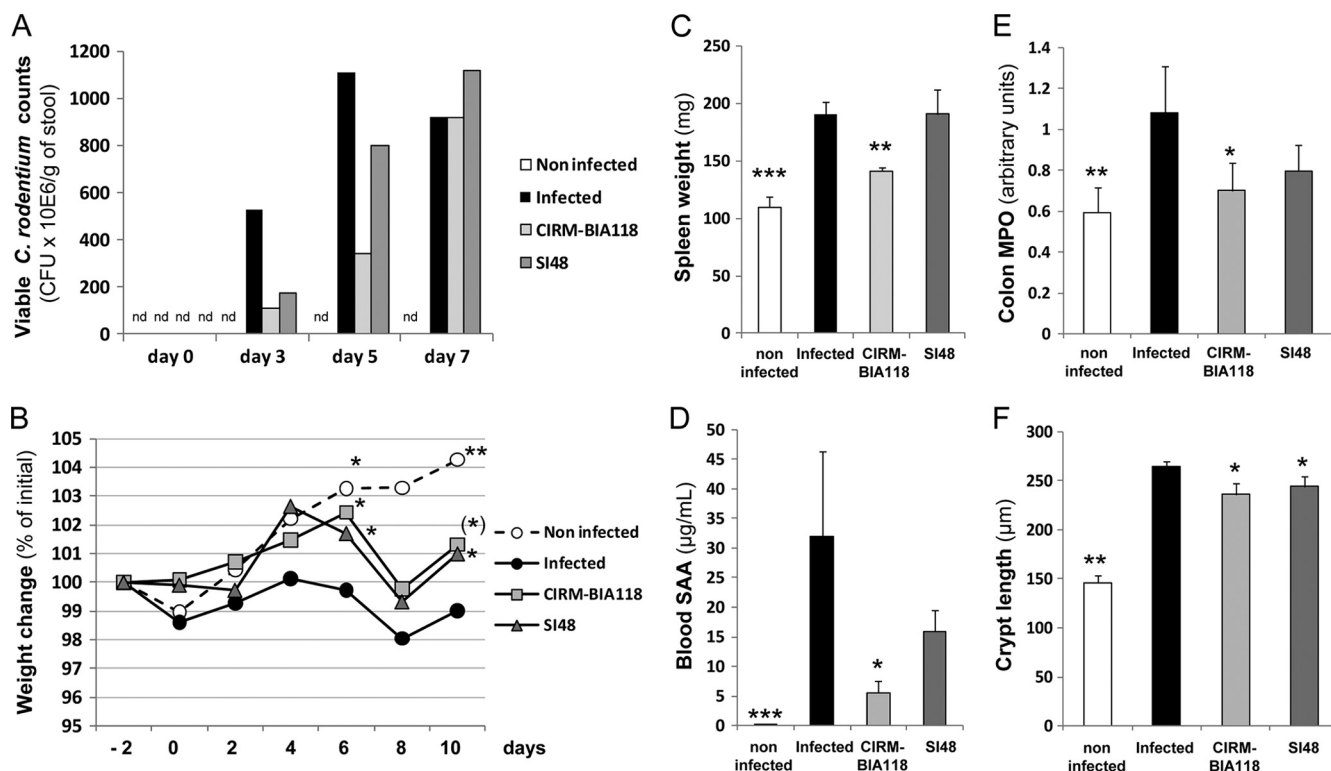
FIG. 3. Protective effect of 5 days of oral treatment with *P. freudenreichii* BIA118 and SI48 (5×10^8 CFU per day) on 10-day *C. rodentium* (strain DBS120)-induced colitis. (A) Time course of pathogen enumeration in pooled feces following the distinct treatments. (B) Weight changes (percentage of initial weight) for healthy control mice (empty circles) and infected control (full circles), BIA118-fed (squares), and SI48-fed mice (triangles). (C to F) Spleen weight (C), blood serum amyloid A protein (mg/ml) (D), colonic MPO activities (E), and crypt length (μm) (F) measured on at least 10 well-orientated sections per animal per group. The results are expressed as mean ± SEM ($n = 8$ mice per group), except for weight changes in panel B, where error bars were eliminated for clarity. nd, not detected. Bars with no asterisks, $0.05 < P < 0.1$; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ versus infected animals by Mann-Whitney U test or the Student t test where appropriate.

length and colonic myeloperoxidase (MPO) activity were also significantly reduced in the *P. freudenreichii* group (Fig. 2D and E, respectively).

In addition, we determined whether preventive treatment with *P. freudenreichii* subspecies could attenuate the severity of colonic injury and inflammation of mice infected with *Citrobacter rodentium*. We used a nonlethal model of resistant BALB/c mice sustaining a discrete and moderate colitis but with quantifiable parameters (12, 32). Groups of eight mice received a 5-day prophylactic oral treatment with either *P. freudenreichii* BIA118 or *P. freudenreichii* SI48 (5×10^8 CFU per day and per mouse) or the corresponding vehicle before a single oral inoculum (1×10^9 CFU) of *C. rodentium* strain DBS120 was administered (29). The counts of the fecal pathogens increased progressively in infected-control mice (Fig. 3A) together with a slight but significant decrease in whole body weight (Fig. 3B) at 10 days postinfection. The colitis-associated markers were also characterized by splenomegaly (assessed by spleen weight) and increased blood serum amyloid A (SAA) levels (Fig. 3C and D). Colons exhibited increased MPO activity (see above) and crypt hyperplasia (assessed by crypt length measurements on histological colon sections) (Fig. 3E and F, respectively). Although both *P. freudenreichii* strains reduced fecal pathogen counts only until day 5, strain *P. freudenreichii* BIA118 significantly, and strain *P. freudenreichii*

SI48 to a lesser extent, lowered most of the parameters measured at the end of the experiment (Fig. 3). It could be argued that such lowering rather evokes a delay of the onset of colitis than a direct anti-inflammatory effect. However, based on previous kinetic studies at days 6 and 12 postinfection (p.i.) (J. Breton, B. Pot, and B. Foligné, unpublished data), we know that the markers investigated are highly correlated with fecal (and cecal) pathogen counts and time; moreover, blood SAA is an early marker that reaches a maximum at day 6 p.i. before it decreases at day 12 p.i. Consequently, beside an anti-infectious effect, such a delay would not reduce SAA levels in probiotic-treated animals. Preventive effects of probiotics against *C. rodentium* were already described for lactobacilli and yeasts (14, 34) but, to our knowledge, never for dairy propionibacteria. Alvarez et al. reported that feeding a *Propionibacterium acidipropionici* supplement to mice prior to *Salmonella typhimurium* (now known as *S. enterica* serovar Typhimurium) administration afforded a partial protection against colonization by the pathogen, measured by a decrease in tissue colonization by *S. typhimurium* and an increase in the mouse survival rate (1). Here, *P. freudenreichii* strains did not affect colonization of *C. rodentium* on the longer term but mainly alleviated inflammatory symptoms linked to the infection.

A key role for surface compounds? We suspected some surface compounds to be involved in the observed immuno-

modulatory effects of dairy propionibacteria. Therefore, in a preliminary attempt to estimate the importance of surface proteins, we removed the surface layer protein(s) by guanidium chloride treatment as described previously (20) and as depicted by scanning electron microscopy (Fig. 1F). This treatment indeed decreased the *in vitro* cytokine induction, turning the strains to display a more proinflammatory profile on human PBMCs (Fig. 1G). Although the importance of surface layer proteins was already observed for a probiotic *Lactobacillus* strain (17), this result needs to be extensively confirmed *in vivo* for different propionibacterial subspecies. Similarly, we can hypothesize that other components such as exopolysaccharides (EPS) may also play an important role in the bacterium-host interaction as reported for other probiotic genera, using both *in vitro* and *in vivo* models (4, 28, 30). The presence of EPS is strain dependent in *P. freudenreichii* (5), and preliminary data based on stimulation of PBMCs indicate a role of *P. freudenreichii* EPS in such interaction (data not shown). Finally, as established for bifidobacteria (11), specific cell-wall-associated proteins which are known to be highly strain dependent in *P. freudenreichii* may also contribute to a certain extent to the distinct immunomodulatory behavior of these bacteria. The now-available genome sequence of *P. freudenreichii* subsp. *shermanii* CIRM-BIA1 (strain included in this study) (7) will offer molecular approaches to further address this question.

Conclusions. In the past, selection of probiotic strains was mainly empirical and based on technological criteria. As probiotic intervention has strain-specific immunomodulatory effects, it is essential to reliably select the most promising candidates for desired health applications. We described before that the differential activation of human PBMCs by bacterial strains can be considered as a predictive tool to identify Gram-positive probiotic strains with a potential anti-inflammatory effect *in vivo* (9). Here we compared the immune-based biodiversity of a set of first 10, then 5 dairy propionibacteria together with established probiotic reference strains and found an overall characteristic pattern with high levels of IL-10 and very low induction of IL-12, TNF-α, and IFN-γ. In agreement with these profiles we obtained significant *in vivo* protection against colitis in mice, suggesting that selected strains of dairy propionibacteria and/or fermented dairy products containing these bacteria can possibly play a role in a diet designed to prevent and/or limit the severity of IBD in humans. Moreover, experiments with *C. rodentium* infection in mice proved similar efficacy in the context of infectious disease. Further mechanistic studies based on the *Propionibacterium* envelope are required in order to substantiate and select such strains for clinical investigations.

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